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(54) Title: HUMAN G-PROTEIN COUPLED RECEPTOR

(57) Abstract

Human ATP receptor polypeptides and DNA (RNA) encoding such polypeptides and a procedure for producing such polypeptides by recombinant techniques is disclosed. Also disclosed are methods for utilizing such polypeptides for identifying antagonists and agonists to such polypeptides and methods of using the agonists and antagonists therapeutically to treat conditions related to the under-expression and over-expression of the ATP receptor polypeptides. Also disclosed are diagnostic methods for detecting a mutation in the ATP receptor nucleic acid sequences and detecting a level of the soluble form of the receptors in a sample derived from a host.

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# HUMAN G-PROTEIN COUPLED RECEPTOR

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. The polypeptide of the present invention is a human 7-transmembrane G-protein coupled receptor. More particularly, the 7-transmembrane receptor has been putatively identified as a human ATP receptor. The invention also relates to inhibiting the action of such polypeptides.

It is well established that many medically significant biological processes are mediated by proteins participating in signal transduction pathways that involve G-proteins and/or second messengers, e.g., cAMP (Lefkowitz, Nature, 351:353-354 (1991)). Herein these proteins are referred to as proteins participating in pathways with G-proteins or PPG proteins. Some examples of these proteins include the G-Protein Coupled (GPC) receptors, such as those for adrenergic agents and dopamine (Kobilka, B.K., et al., PNAS, 84:46-50 (1987); Kobilka, B.K., et al., Science, 238:650-656 (1987); Bunzow, J.R., et al., Nature, 336:783-787 (1988)), G-proteins themselves, effector proteins, e.g., phospholipase C, adenyl cyclase, and phosphodiesterase, and actuator proteins, e.g., protein

kinase A and protein kinase C (Simon, M.I., et al., Science, 252:802-8 (1991)).

For example, in one form of signal transduction, the effect of hormone binding is activation of an enzyme, adenylate cyclase, inside the cell. Enzyme activation by hormones is dependent on the presence of the nucleotide GTP, and GTP also influences hormone binding. A G-protein connects the hormone receptors to adenylate cyclase. G-protein was shown to exchange GTP for bound GDP when activated by hormone receptors. The GTP-carrying form then binds to an activated adenylate cyclase. Hydrolysis of GTP to GDP, catalyzed by the G-protein itself, returns the G-protein to its basal, inactive form. Thus, the G-protein serves a dual role, as an intermediate that relays the signal from receptor to effector, and as a clock that controls the duration of the signal.

Extracellular nucleotides, such as ATP, can influence many biological functions, including platelet aggregation, vascular tone, cell division, cardiac and skeletal muscle contraction, as well as peripheral and central neurotransmission. Gordon, J.L., *Biochem. J.* 233:309-319 (1986). These extracellular actions of ATP are mediated through purinergic receptors that have been classified by Burnstock G. ((1978) *Cell Membrane receptors for Drugs and Hormones: A multidisciplinary approach* (Straub, R.W. and Bolis, L. Eds) pp. 107-118, Raven Press, New York)) as $P_2$ receptors. $P_2$ receptors were subclassified into $P_2x$ and $P_2y$, based on the order of potency of ATP analogues to increase smooth muscle tension (Burnstock G. and Kennedy, C., *Gen. Pharmacol.* 16:433-440 (1985)). More recently, the number of purinergic receptor subtypes has been further expanded (Burnstock G., *Cell Membrane receptors for Drugs and Hormones: A multidisciplinary approach* (Straub, R.W. and Bolis, L. Eds) pp. 107-118, Raven Press, New York (1978)) with the addition of the $P_2t$ receptor subtype that is stimulated by ADP by antagonized by ATP, $P_2u$ receptor subtype (Murrin, R.J.A. and Boparder, M.R. *Mol. Pharmacol.* 41:561-568 (1992)) that recognizes UTP as well as ATP, and

$P_2z$ receptor subtype that recognizes $ATP^{4-}$ and causes permeabilization of the cell membrane.

In PC12 cells, which have many neuronal characteristics and have been used as a neuronal model, extracellular ATP produced a rapid and transient increase in intracellular calcium level and a release of neurotransmitter (Fasolato, C. Pizzo, P. and Pozzan, T., *J. Biol. Chem.* 265:20351-20355 (1990); Sela, D., Ram, E. and Atlas, D. *J. Biol. Chem.* 266:17990-17994 (1991); Majid, M.A., Okajima, F. and Kondo, Y., *Biochem. Biophy. Acta* 1136:283-289 (1992)). The purinergic $P_2$ receptor on the PC12 cells enables this interaction. A new $P_2$ receptor was discovered based on measurements of ATP-stimulated increases in $[Ca^{2+}]$, and the properties of $\alpha$-$^{35}$S-ATP binding (Kim, W. K. and Rabin, R.A., J. Bio. Chem., 269(9):6471-6477 (1994).

The role of nucleotides in metabolism is well established, but recognition of their potential importance as extracellular transmitters, regulators or modulators has been very recent. Extracellular ATP has been shown to induce a variety of responses, such as stimulation of inositol phospholipid turnover and activation of membrane conductances in many cell types and preparations (Burnstock, G., *Nucleosides Nucleotides* **10**:917-930 (1991); Bean, B.P., *Trends Pharmacol Sci.* **13**:87-90 (1992); Edwards F.A., Gibb, A.J. & Colquhoun, D., *Nature (London)* **359**:144-147 (1992); Kastritsis, C.H.C., Salm, A.K. & McCarthy, K., *J. Neurochem.* **58**:1277-1284 (1992)). These responses are now known to be mediated by a family of ATP receptors, designated $P_2$ purinoceptors (Burnstock, G. & Kennedy, C., *Gen. Pharmacol.* **16**:433-440 (1985); Gordon, J.L., *Biochem. J.* **233**:309-319 (1986); Dubyak, G.R., *Am. J. Respir. Cell Mol. Biol.* **4**:295-300 (1991)), some of which have been cloned (Lustig, K.D., Shiau, A.K., Brake, A.J. & Julius, D., *Proc. Natl. Acad. Sci, USA* **90**:5113-5117 (1993); Webb, T.E., Simon, J., Krishek, B.J., Bateson, A.N., Smart, T.G., King, B.F., Burnstock, G. & Barnard, E.A., *FEBS Lett* **324**:219-225 (1993); Brake, A.J., Wagenbach, M.J. & Julius,

D., *Nature (London)* 371:519-523 (1994); Valera, S., Hussy, N., Evans, R.J., Adami, N., North, R.A., Surprenant, A. & Buell, G., *Nature (London)* 371:516-519 (1994)).

ATP has been shown to stimulate inositol phosphate accumulation and intracellular $Ca^{2+}$ metabolism in pituitary cell cultures (Davidson, J.S., Wakefield, I.K., Sohnius, U., van der Merwe, P.A. & Millar, R.P., *Endocrinology* 126:80-87 (1990)), and data from fetal hypothalamic neuron cultures suggests that ATP has a possible regulatory role in the neuroendocrine system (Chen, Z.P., Levy, A. & Lightman, S.L., *Brain Res.* 641:249-256 (1994)). ATP and UTP act on ATP receptors to provoke a rapid and dramatic increase in cytosolic $Ca^{2+}$ in pituitary gonadotropin-release hormone (GnRH)-responsive cells (Chen, Z.P., Levy, A., McArdle, C.A. & Lightman, S.L., *Endocrinology* 135:1280-1283 (1994)). ATP receptors can also mediate significant release of pituitary gonadotropin and that ATP can be exocytotically released from pituitary cells.

In accordance with one aspect of the present invention, there are provided novel mature receptor polypeptides as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof. The receptor polypeptides of the present invention are of human origin.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding the receptor polypeptides of the present invention, including mRNAs, cDNAs, genomic DNA as well as antisense analogs thereof and biologically active and diagnostically or therapeutically useful fragments thereof.

In accordance with another aspect of the present invention there is provided an isolated nucleic acid molecule encoding a mature polypeptide expressed by the human cDNA contained in ATCC Deposit No. 97333.

In accordance with a further aspect of the present invention, there are provided processes for producing such receptor polypeptides by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host

cells, containing nucleic acid sequences encoding the receptor polypeptides of the present invention, under conditions promoting expression of said polypeptides and subsequent recovery of said polypeptides.

In accordance with yet a further aspect of the present invention, there are provided antibodies against such receptor polypeptides.

In accordance with another aspect of the present invention there are provided methods of screening for compounds which bind to and activate or inhibit activation of the receptor polypeptides of the present invention.

In accordance with still another embodiment of the present invention there are provided processes of administering compounds to a host which bind to and activate the receptor polypeptide of the present invention which mediate physiological response in pituitary cells and to stimulate luteinizing hormone release.

In accordance with another aspect of the present invention there is provided a method of administering the receptor polypeptides of the present invention via gene therapy to treat conditions related to underexpression of the polypeptides or underexpression of a ligand to the receptor polypeptide.

In accordance with still another embodiment of the present invention there are provided processes of administering compounds to a host which bind to and inhibit activation of the receptor polypeptides of the present invention which are useful in the prevention and/or treatment of arterial thrombosis, including angina, myocardial infarction and stroke, thrombolysis, angioplasty and cystic fibrosis.

In accordance with yet another aspect of the present invention, there are provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to the polynucleotide sequences of the present invention.

In accordance with still another aspect of the present invention, there are provided diagnostic assays for

detecting diseases related to mutations in the nucleic acid sequences encoding such polypeptides and for detecting an altered level of the soluble form of the receptor polypeptides.

In accordance with yet a further aspect of the present invention, there are provided processes for utilizing such receptor polypeptides, or polynucleotides encoding such polypeptides, for in vitro purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 shows the cDNA sequence and the corresponding deduced amino acid sequence of the G-protein coupled receptor of the present invention. The standard one-letter abbreviation for amino acids is used. Sequencing was performed using a 373 Automated DNA sequencer (Applied Biosystems, Inc.).

Figure 2 illustrates the amino acid sequence homology between the polypeptide of the present invention (top line) (SEQ ID NO:2) and a murine $P_2u$ receptor (SEQ ID NO:9).

In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2).

The polynucleotide of this invention was discovered in a cDNA library derived from a human placenta. It is structurally related to the G protein-coupled receptor family and is a putative member of that family. It contains an open reading frame encoding a protein of 334 amino acid residues. The protein exhibits the highest degree of homology to a murine $P_2u$ receptor with 29.787 % identity and 51.064 % similarity over the entire amino acid stretch.

In accordance with another aspect of the present invention there are provided isolated polynucleotides encoding a mature polypeptide expressed by the human cDNA contained in ATCC Deposit No. 97333 deposited with the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, USA, on November 6, 1995. The deposited material is an a pBluescript SK(-) plasmid which contains the full-length ATP receptor cDNA.

The deposit(s) have been made under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. The strain will be irrevocably and without restriction or condition released to the public upon the issuance of a patent. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted. References to "polynucleotides" throughout this specification includes the DNA of the deposit referred to above.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figure 1 (SEQ ID NO:1) or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature polypeptide as the DNA of Figure 1 (SEQ ID NO:1).

The polynucleotide which encodes for the mature polypeptide of Figure 1 (SEQ ID NO:2) may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 1 (SEQ ID NO:2). The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in Figure 1 (SEQ ID NO:2) as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the polypeptide of Figure 1 (SEQ ID NO:2). Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in Figure 1 (SEQ ID NO:1). As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The polynucleotides may also encode for a soluble form of the ATP receptor polypeptide which is the extrac llular portion of the polypeptide which has been cleaved from the

TM and intracellular domain of the full-length polypeptide of the present invention.

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be, for example, a hexa-histidine tag supplied by a pQE vector (Qiagen, Inc.) to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which either retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs of Figure 1 (SEQ ID NO:1), i.e. function as a soluble ATP receptor by retaining the ability to bind the ligands for the receptor even though the polypeptide does not function as a membrane bound ATP receptor, for example, by eliciting a second messenger response.

Alternatively, the polynucleotides may have at least 15 bases, preferably at least 30 bases and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which have an identity thereto,

as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotide of SEQ ID NO: 1, or for variants thereof, for example, for recovery of the polynucleotide or as a diagnostic probe or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least a 90% identity and more preferably at least a 95% identity to a polynucleotide which encodes the polypeptide of SEQ ID NO:2 and polynucleotides complementary thereto as well as portions thereof, which portions have at least 30 consecutive bases and preferably at least 50 consecutive bases and to polypeptides encoded by such polynucleotides.

Fragments of the genes may be employed as a hybridization probe for a cDNA library to isolate other genes which have a high sequence similarity to the genes of the present invention, or which have similar biological activity. Probes of this type are at least 15 bases, preferably at least 30 bases and most preferably at least 50 bases or more. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene of the present invention including regulatory and promoter regions, exons and introns. An example of a screen of this type comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the genes of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The present invention further relates to an ATP receptor polypeptide which has the deduced amino acid sequence of Figure 1 (SEQ ID NO:2), as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figure 1 (SEQ ID NO:2), means a polypeptide which either retains substantially the

same biological function or activity as such polypeptide, i.e. functions as an ATP receptor, or retains the ability to bind the ligand for the receptor even though the polypeptide does not function as a G-protein coupled receptor, for example, a soluble form of the receptor.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The fragment, derivative or analog of the polypeptide of Figure 1 (SEQ ID NO:2) may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide which are employed for purification of the mature polypeptide or a proprotein sequence or (v) one in which a fragment of the polypeptide is soluble, i.e. not membrane bound, yet still binds ligands to the membrane bound receptor. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and still more preferably at

least 95% similarity (still more preferably at least 95% identity) to the polyp ptide of SEQ ID NO:2 and also includes portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis, therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region "leader and trailer" as well as intervening sequences (introns) between individual coding segments (exons).

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli lac or trp, the phage lambda $P_L$ promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also

contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila and Spodoptera Sf9; animal cells such as CHO, COS or Bowes melanoma; adenovirus; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pbs, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV,

pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are PKK232-8 and PCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda $P_R$, $P_L$ and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. (Davis, L., Dibner, M., Battey, I., Basic Methods in Molecular Biology, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various

species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well know to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the

SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The ATP receptor polypeptides can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. Polypeptides of the invention may also include an initial methionine amino acid residue.

The polynucleotides and polypeptides of the present invention may be employed as research reagents and materials for discovery of treatments and diagnostics to human disease.

The ATP receptor polypeptide of the present invention may be employed in a process for screening for compounds which activate (agonists) or inhibit activation (antagonists) of the receptor polypeptide of the present invention .

In general, such screening procedures involve providing appropriate cells which express the receptor polypeptide of the present invention on the surface thereof. Such cells include cells from mammals, yeast, drosophila or *E. Coli*. In particular, a polynucleotide encoding the receptor of the present invention is employed

to transfect cells to thereby express the polypeptide of the present invention. The expressed receptor is then contacted with a test compound to observe binding, stimulation or inhibition of a functional response.

One such screening procedure involves the use of melanophores which are transfected to express the polypeptide of the present invention. Such a screening technique is described in PCT WO 92/01810 published February 6, 1992.

Thus, for example, such assay may be employed for screening for a compound which inhibits activation of the receptor polypeptide of the present invention by contacting the melanophore cells which encode the receptor with both the receptor ligand and a compound to be screened. Inhibition of the signal generated by the ligand indicates that a compound is a potential antagonist for the receptor, i.e., inhibits activation of the ATP receptor.

The screen may be employed for determining a compound which activates the receptor by contacting such cells with compounds to be screened and determining whether such compound generates a signal, i.e., activates the receptor.

Other screening techniques include the use of cells which express the polypeptide of the present invention (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation, for example, as described in Science, volume 246, pages 181-296 (October 1989). For example, compounds may be contacted with a cell which expresses the receptor polypeptide of the present invention and a second messenger response, e.g. signal transduction or pH changes, may be measured to determine whether the potential compound activates or inhibits the receptor.

Another such screening technique involves introducing RNA encoding the polypeptide of the present invention into *Xenopus* oocytes to transiently express the receptor. The receptor oocytes may then be contacted with the receptor ligand and a compound to be screened, followed by detection of inhibition or activation of a calcium signal in the case

of screening for compounds which are thought to inhibit activation of the receptor.

Another screening technique involves expressing the polypeptide of the present invention in which the polypeptide is linked to a phospholipase C or D. As representative examples of such cells, there may be mentioned endothelial cells, smooth muscle cells, embryonic kidney cells, etc. The screening may be accomplished as hereinabove described by detecting activation of the receptor or inhibition of activation of the receptor from the phospholipase second signal.

Another method involves screening for compounds which inhibit activation of the receptor polypeptide of the present invention antagonists by determining inhibition of binding of labeled ligand to cells which have the receptor on the surface thereof. Such a method involves transfecting a eukaryotic cell with DNA encoding the ATP receptor polypeptide such that the cell expresses the ATP receptor on its surface and contacting the cell with a compound in the presence of a labeled form of a known ligand. The ligand can be labeled, e.g., by radioactivity. The amount of labeled ligand bound to the ATP receptor is measured, e.g., by measuring radioactivity of the receptors. If the compound binds to the ATP receptor as determined by a reduction of labeled ligand which binds to the ATP receptor, the binding of labeled ligand to the ATP receptor is inhibited.

G-protein coupled receptors are ubiquitous in the mammalian host and are responsible for many biological functions, including many pathologies. Accordingly, it is desirous to find compounds and drugs which stimulate the ATP receptor polypeptide of the present invention on the one hand and which can inhibit its function on the other hand.

For example, compounds which activate G-protein coupled receptors may be employed for therapeutic purposes, such as the treatment of asthma, Parkinson's disease, acute

heart failure, hypotension, urinary retention, and osteoporosis.

In general, compounds which inhibit activation of G-protein coupled receptors may be employed for a variety of therapeutic purposes, for example, for the treatment of hypertension, angina pectoris, myocardial infarction, ulcers, asthma, allergies, benign prostatic hypertrophy and psychotic and neurological disorders, including schizophrenia, manic excitement, depression, delirium, dementia or severe mental retardation, dyskinesias, such as Huntington's disease or Gilles dila Tourett's syndrome, reversing endogenous anorexia and in the control of bulimia, among others.

An antibody may antagonize an ATP receptor of the present invention, or in some cases an oligopeptide, which bind to the ATP receptor but does not elicit a second messenger response such that the activity of the ATP receptor is prevented. Antibodies include anti-idiotypic antibodies which recognize unique determinants generally associated with the antigen-binding site of an antibody. Potential antagonist compounds also include proteins which are closely related to the ligand of the ATP receptors, i.e. a fragment of the ligand, which have lost biological function and when binding to the ATP receptor, elicit no response.

An antisense construct prepared through the use of antisense technology, may be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360

(1991)), thereby preventing transcription and the production of the ATP receptor. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of mRNA molecules into G-protein coupled receptor (antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the ATP receptor.

A small molecule which binds to the ATP receptor, making it inaccessible to ligands such that normal biological activity is prevented, for example small peptides or peptide-like molecules, may also be used to inhibit activation of the ATP receptor polypeptide of the present invention.

A soluble form of the ATP receptor, e.g. a fragment of the receptor, may be employed to inhibit activation of the receptor by binding to the ligand and preventing the ligand from interacting with membrane bound ATP receptors.

In general, antagonists for ATP receptors which are determined by screening procedures may be employed for a variety of therapeutic purposes. For example, such antagonists have been employed for treatment of hypertension, angina pectoris, myocardial infarction, ulcers, asthma, allergies, psychoses, depression, migraine, vomiting, and benign prostatic hypertrophy.

The antagonists specific to the ATP receptor of the present invention may be employed to treat arterial thrombosis including angina, myocardial infarction and stroke, thrombolysis, angioplasty and cystic fibrosis. The antagonists may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinafter described.

Agonists for G-protein coupled receptors are also useful for therapeutic purposes, such as the treatment of asthma, Parkinson's disease, acute heart failure, hypotension, urinary retention, and osteoporosis.

The agonists for the receptor of the present invention may be employed to treat chronic bronchitis and to mediate the physiological response in pituitary cells, particularly gonadotrope cells including a luteinizing hormone release.

The antagonist or agonist compounds may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the compound, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the pharmaceutical compositions of the present invention may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, the pharmaceutical compositions will be administered in an amount of at least about 10 μg/kg body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10 μg/kg to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

The ATP receptor polypeptides and compounds which are polypeptides, may also be employed in accordance with the

present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus which may be used to engineer cells in vivo after combination with a suitable delivery vehicle.

Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or

any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or hetorologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the β-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the genes encoding the polypeptides.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, ψ-2, ψ-AM, PA12, T19-14X, VT-19-17-H2, ψCRE, ψCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

The present invention also provides a method for determining whether a ligand not known to be capable of binding to an ATP receptor can bind to such receptor which comprises contacting a mammalian cell which expresses an ATP receptor with the ligand under conditions permitting binding of ligands to an ATP receptor, detecting the presence of a ligand which binds to the receptor and thereby determining whether the ligand binds to the receptor. The systems hereinabove described for determining agonists and/or antagonists may also be employed for determining ligands which bind to the receptor.

This invention also provides a method of detecting expression of an ATP receptor polypeptide of the present invention on the surface of a cell by detecting the presence of mRNA coding for the receptor which comprises obtaining total mRNA from the cell and contacting the mRNA so obtained with a nucleic acid probe comprising a nucleic acid molecule of at least 10 nucleotides capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding the receptor under hybridizing conditions, detecting the presence of mRNA hybridized to the probe, and thereby detecting the expression of the receptor by the cell.

The present invention also provides a method for identifying receptors related to the receptor polypeptides

of the present invention. These related receptors may be identified by homology to an ATP receptor polypeptide of the present invention, by low stringency cross hybridization, or by identifying receptors that interact with related natural or synthetic ligands and or elicit similar behaviors after genetic or pharmacological blockade of the ATP receptor polypeptides of the present invention.

The present invention also contemplates the use of the genes of the present invention as a diagnostic, for example, some diseases result from inherited defective genes. These genes can be detected by comparing the sequences of the defective gene with that of a normal one. Subsequently, one can verify that a "mutant" gene is associated with abnormal receptor activity. In addition, one can insert mutant receptor genes into a suitable vector for expression in a functional assay system (e.g., colorimetric assay, expression on MacConkey plates, complementation experiments, in a receptor deficient strain of HEK293 cells) as yet another means to verify or identify mutations. Once "mutant" genes have been identified, one can then screen population for carriers of the "mutant" receptor gene.

Individuals carrying mutations in the gene of the present invention may be detected at the DNA level by a variety of techniques. Nucleic acids used for diagnosis may be obtained from a patient's cells, including but not limited to such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki, et al., Nature, 324:163-166 1986) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complimentary to the nucleic acid of the instant invention can be used to identify and analyze mutations in the gene of the present invention. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radio labeled

RNA of the invention or alternatively, radio labeled antisense DNA sequences of the invention. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures. Such a diagnostic would be particularly useful for prenatal or even neonatal testing. Sequence differences between the reference gene and "mutants" may be revealed by the direct DNA sequencing method. In addition, cloned DNA segments may be used as probes to detect specific DNA segments. The sensitivity of this method is greatly enhanced when combined with PCR. For example, a sequence primer is used with double stranded PCR product or a single stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures with radio labeled nucleotide or by an automatic sequencing procedure with fluorescent-tags.

Genetic testing based on DNA sequence differences may be achieved by detection of alterations in the electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Sequences changes at specific locations may also be revealed by nucleus protection assays, such RNase and S1 protection or the chemical cleavage method (e.g. Cotton, et al., PNAS, USA, 85:4397-4401 1985).

In addition, some diseases are a result of, or are characterized by changes in gene expression which can be detected by changes in the mRNA. Alternatively, the genes of the present invention can be used as a reference to identify individuals expressing a decrease of functions associated with receptors of this type.

The present invention also relates to a diagnostic assay for detecting altered levels of soluble forms of the ATP receptor polypeptides of the present invention in various tissues. Assays used to detect levels of the soluble receptor polypeptides in a sample derived from a host are well known to those of skill in the art and include radioimmunoassays, competitive-binding assays, Western blot analysis and preferably as ELISA assay.

An ELISA assay initially comprises preparing an antibody specific to antigens of the ATP receptor polypeptides, preferably a monoclonal antibody. In addition a reporter antibody is prepared against the monoclonal antibody. To the reporter antibody is attached a detectable reagent such as radioactivity, fluorescence or in this example a horseradish peroxidase enzyme. A sample is now removed from a host and incubated on a solid support, e.g. a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein such as bovine serum albumin. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to any ATP receptor proteins attached to the polystyrene dish. All unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is now placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to ATP receptor proteins. Unattached reporter antibody is then washed out. Peroxidase substrates are then added to the dish and the amount of color developed in a given time period is a measurement of the amount of ATP receptor proteins present in a given volume of patient sample when compared against a standard curve.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the cDNA is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60 bases. For a review of this technique, see Verma et al., Human Chromosomes: a Manual of Basic Techniques, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and

unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in Monoclonal

Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The above-described antibodies may be employed to isolate the polypeptide of the present invention by attachment of the antibody to a solid support and performing affinity chromatography by passing the polypeptide desired to be purified over the column and recovering the purified polypeptide.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 μg of plasmid or DNA fragment is used with about 2 units of enzyme in about 20

μl of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μg of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. *et al.*, Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units to T4 DNA ligase ("ligase") per 0.5 μg of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, F. and Van der Eb, A., Virology, 52:456-457 (1973).

## Example 1

### Bacterial Expression and Purification of ATP receptor

The DNA sequence encoding ATP receptor, ATCC # 97333, is initially amplified using PCR oligonucleotide primers corresponding to the 5' sequences of the processed ATP receptor protein and the vector sequences 3' to the ATP

receptor gene. Additional nucleotides corresponding to ATP receptor are added to the 5' and 3' sequences respectively. The 5' oligonucleotide primer has the sequence 5' CCGCAGATCAATGCTGGGGATCATGGCA 3' (SEQ ID NO:3) contains a BglII restriction enzyme site followed by 18 nucleotides of ATP receptor coding sequence starting from the presumed terminal amino acid of the processed protein. The 3' sequence 5' CGGCTCTAGATCACTTTTCTCTGAATGA 3' (SEQ ID NO:4) contains complementary sequences to an XbaI site and is followed by 18 nucleotides of ATP receptor and to a vector sequence located 3' to the ATP receptor DNA insert. The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE-9 (Qiagen, Inc. Chatsworth, CA). pQE-9 encodes antibiotic resistance (Amp'), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. pQE-9 is then digested with BglII and XbaI. The amplified sequences are ligated into pQE-9 and are inserted in frame with the sequence encoding for the histidine tag and the RBS. The ligation mixture is then used to transform E. coli strain M15/rep 4 (Qiagen, Inc.) by the procedure described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989). M15/rep4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan'). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies are selected. Plasmid DNA is isolated and confirmed by restriction analysis. Clones containing the desired constructs are grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells are grown to an optical density 600 ($O.D._{600}$) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") is then added to a final concentration of 1 mM. IPTG induces by

inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells are grown an extra 3 to 4 hours. Cells are then harvested by centrifugation. The cell pellet is solubilized in the chaotropic agent 6 Molar Guanidine HCl. After clarification, solubilized ATP receptor is purified from this solution by chromatography on a Nickel-Chelate column under conditions that allow for tight binding by proteins containing the 6-His tag (Hochuli, E. et al., J. Chromatography 411:177-184 (1984)). ATP receptor is eluted from the column in 6 molar guanidine HCl pH 5.0 and for the purpose of renaturation adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 mmolar glutathione (reduced) and 2 mmolar glutathione (oxidized). After incubation in this solution for 12 hours the protein is dialyzed to 10 mmolar sodium phosphate.

Example 2

Cloning and expression of ATP receptor using the baculovirus expression system

The DNA sequence encoding the full length ATP receptor protein, ATCC # 97333, is amplified using PCR oligo-nucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer has the sequence 5' CGGGATCCCTCCATGCTGGGG ATCATGGCA 3' (SEQ ID NO:5) and contains a BamHI restriction enzyme site (in bold) followed by 4 nucleotides resembling an efficient signal for the initiation of translation in eukaryotic cells (Kozak, M., J. Mol. Biol., 196:947-950 (1987) which is just behind the first 18 nucleotides of the ATP receptor gene (the initiation codon for translation "ATG" is underlined).

The 3' primer has the sequence 5' CGGGATCCGCTCACTTTTCTCT GAATGA 3' (SEQ ID NO:6) and contains the cleavage site for the restriction endonuclease BamHI (in bold) and 18 nucleotides complementary to the 3' non-translated sequence of the ATP receptor gene. The amplified sequences are

isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment is then digested with the endonuclease BamHI and purified again on a 1% agarose gel. This fragment is designated F2.

The vector pRG1 (modification of pVL941 vector, discussed below) is used for the expression of the ATP receptor protein using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by the recognition sites for the restriction endonuclease BamHI. The polyadenylation site of the simian virus (SV)40 is used for efficient polyadenylation. For an easy selection of recombinant virus the beta-galactosidase gene from E.coli is inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by viral sequences for the cell-mediated homologous recombination of co-transfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pRG1 such as pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., Virology, 170:31-39).

The plasmid is digested with the restriction enzymes BamHI and dephosphorylated using calf intestinal phosphatase by procedures known in the art. The DNA is then isolated from a 1% agarose gel using the commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated V2.

Fragment F2 and the dephosphorylated plasmid V2 are ligated with T4 DNA ligase. E. coli HB101 cells are then transformed and bacteria identified that contained the plasmid (pBac ATP receptor) with the ATP receptor gene using the enzyme BamHI. The sequence of the cloned fragment is confirmed by DNA sequencing.

5 µg of the plasmid pBac ATP receptor is co-transfected with 1.0 µg of a commercially available linearized baculovirus ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofection method (Felgner et al. Proc. Natl. Acad. Sci. USA, 84:7413-7417 (1987)).

1µg of BaculoGold™ virus DNA and 5 µg of the plasmid pBac ATP receptor are mixed in a sterile well of a microtiter plate containing 50 µl of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 µl Lipofectin plus 90 µl Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for 5 hours at 27°C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation continued at 27°C for four days.

After four days the supernatant is collected and a plaque assay performed similar as described by Summers and Smith (supra). As a modification an agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution the virus is added to the cells and blue stained plaques are picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses is then resuspended in an Eppendorf tube containing 200 µl of Grace's medium. The agar is removed by a brief centrifugation and the supernatant containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the

supernatants of these culture dishes are harvested and then stored at 4°C.

Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus V-ATP receptor at a multiplicity of infection (MOI) of 2. Six hours later the medium is removed and replaced with SF900 II medium minus methionine and cysteine (Life Technologies Inc., Gaithersburg). 42 hours later 5 $\mu$Ci of $^{35}$S-methionine and 5 $\mu$Ci $^{35}$S cysteine (Amersham) are added. The cells are further incubated for 16 hours before they are harvested by centrifugation and the labelled proteins visualized by SDS-PAGE and autoradiography.

Example 3

Expression of Recombinant ATP receptor in COS cells

The expression of plasmid, ATP receptor HA is derived from a vector pcDNAI/Amp (Invitrogen) containing: 1) SV40 origin of replication, 2) ampicillin resistance gene, 3) E.coli replication origin, 4) CMV promoter followed by a polylinker region, an SV40 intron and polyadenylation site. A DNA fragment encoding the entire ATP receptor precursor and a HA tag fused in frame to its 3' end is cloned into the polylinker region of the vector, therefore, the recombinant protein expression is directed under the CMV promoter. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein as previously described (I. Wilson, H. Niman, R. Heighten, A Cherenson, M. Connolly, and R. Lerner, 1984, Cell 37:767, (1984)). The infusion of HA tag to the target protein allows easy detection of the recombinant protein with an antibody that recognizes the HA epitope.

The plasmid construction strategy is described as follows:

The DNA sequence encoding ATP receptor, ATCC # 97333, is constructed by PCR cloned using two primers: the 5' primer 5' GTCCAAGCTTGCCACCATGCTGGGGATCATGGCA 3' (SEQ ID NO:7) contains a HindIII site (in bold) followed by 18

nucleotides of ATP receptor coding sequence starting from the initiation codon; the 3' sequence 5' CTAGCTCGAGTCAAGCGTAGTCTGGGACGTCGTA TGGGTAGCACTTTTCTCTGAATGAAAG 3' (SEQ ID NO:8) contains complementary sequences to an XhoI site (in bold), translation stop codon, HA tag and the last 18 nucleotides of the ATP receptor coding sequence (not including the stop codon). Therefore, the PCR product contains a HindIII site, ATP receptor coding sequence followed by HA tag fused in frame, a translation termination stop codon next to the HA tag, and an XhoI site. The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with HindIII and XhoI restriction enzyme and ligated. The ligation mixture is transformed into E. coli strain SURE (available from Stratagene Cloning Systems, La Jolla, CA 92037) the transformed culture is plated on ampicillin media plates and resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis for the presence of the correct fragment. For expression of the recombinant ATP receptor, COS cells are transfected with the expression vector by DEAE-DEXTRAN method (J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989)). The expression of the ATP receptor HA protein is detected by radiolabelling and immunoprecipitation method (E. Harlow, D. Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, (1988)). Cells are labelled for 8 hours with $^{35}$S-cysteine two days post transfection. Culture media is then collected and cells are lysed with detergent (RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50mM Tris, pH 7.5) (Wilson, I. et al., Id. 37:767 (1984)). Both cell lysate and culture media are precipitated with an HA specific monoclonal antibody. Proteins precipitated are analyzed on 15% SDS-PAGE gels.

## Example 4

## Expression via Gene Therapy

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin, is added. This is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al, DNA, 7:219-25 (1988) flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention is amplified using PCR primers which correspond to the 5' and 3' end sequences respectively. The 5' primer containing an EcoRI site and the 3' primer further includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is used to transform bacteria HB101, which are then plated onto agar-containing kanamycin for the purpose of confirming that the vector had the gene of interest properly inserted.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS),

penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells are transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a polynucleotide having at least a 70% identity to a member selected from the group consisting of:

(a) a polynucleotide encoding a polypeptide comprising an amino acid sequence set forth in Figure 1;

(b) a polynucleotide which is complementary to the polynucleotide of (a); and

(c) a polynucleotide comprising at least 30 bases of the polynucleotide of (a) or (b).

2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.

3. The polynucleotide of Claim 1 wherein said polynucleotide is RNA.

4. The polynucleotide of Claim 1 encoding a polypeptide set forth in Figure 1.

5. The polynucleotide of Claim 2 comprising a nucleotide sequence set forth in Figure 1.

6. The polynucleotide of Claim 2 comprising from nucleotide 93 to nucleotide 1094 set forth in Figure 1.

7. The polynucleotide of Claim 1 encoding a soluble form of the polypeptide of Figure 1.

8. An isolated polynucleotide comprising a polynucleotide which is at least 70% identical to a member selected from the group consisting of:

(a) a polynucleotide encoding the same mature polypeptide expressed by the human cDNA contained in ATCC Deposit No. 97333;

(b) a polynucleotide which is complementary to the polynucleotide of (a); and

(c) a polynucleotide comprising at least 30 bases of the polynucleotide of (a) or (b).

9. The isolated polynucleotide of Claim 8 wherein said polynucleotide encodes the same mature polypeptide expressed by the human cDNA contained in ATCC Deposit No. 97333.

10. The isolated polynucleotide of Claim 8 wherein said polynucleotide encodes a polypeptide expressed by the human cDNA contained in ATCC Deposit No. 97333.

11. A vector comprising the DNA of Claim 2.

12. A host cell comprising the vector of Claim 11.

13. A process for producing a polypeptide comprising: expressing from the host cell of Claim 12 a polypeptide encoded by the human cDNA contained in the vector.

14. A process for producing a cell comprising: transforming or transfecting the cell with the vector of claim 11 such that the cell expresses a polypeptide encoded by the human cDNA contained in said vector.

15. A polypeptide comprising an amino acid sequence selected from the group consisting of:

(a) a polypeptide which is at least 70% identical to a polypeptide comprising an amino acid sequence set forth in Figure 1; and

(b) a polypeptide comprising at least 30 amino acid residues of the polypeptide of (a).

16. The polypeptide of Claim 15 wherein the polypeptide has an amino acid sequence set forth in Figure 1.

17. An antibody against the polypeptide of claim 15.

18. An agonist to the polypeptide of claim 15.

19. An antagonist against the polypeptide of claim 15.

20. A method for the treatment of a patient having need to activate an ATP receptor comprising: administering to the patient a therapeutically effective amount of the agonist of claim 18.

21. A method for the treatment of a patient having need to inhibit an ATP receptor comprising: administering to the patient a therapeutically effective amount of the antagonist of claim 19.

22. The method of claim 20 wherein said agonist is a polypeptide and a therapeutically effective amount of said agonist is administered by providing to the patient DNA encoding said agonist and expressing said agonist *in vivo*.

23. The method of claim 21 wherein said antagonist is a polypeptide and a therapeutically effective amount of the antagonist is administered by providing to the patient DNA encoding said antagonist and expressing said antagonist *in vivo*.

24. A method for identifying compounds which bind to and activate the polypeptide of claim 15 comprising:

contacting a cell expressing on the surface thereof an ATP receptor, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with a compound under conditions sufficient to permit binding of the compound to the receptor; and

identifying if the compound binds to the receptor by detecting the signal produced by said second component.

25. A method for identifying compounds which bind to and inhibit activation of the polypeptide of claim 15 comprising:

contacting a cell expressing on the surface thereof an ATP receptor, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with an analytically detectable ligand known to bind to the receptro and a compound to be screened under conditions to permit binding to the receptor; and

determining whether the compound inhibits activation of the receptor by detecting the absence of a signal generated from the interaction of the ligand with the receptor.

26. A process for diagnosing a disease or a susceptibility to a disease related to an under-expression of the polypeptide of claim 15 comprising:

determining a mutation in the nucleic acid sequence encoding said polypeptide.

27. The polypeptide of Claim 15 wherein the polypeptide is a soluble fragment of the polypeptide and is capable of binding a ligand to the polypeptide.

28. A diagnostic process comprising:

analyzing for the presence of the polypeptide of claim 27 in a sample derived from a host.

FIG. 1A

```
          10                  30                  50
CCAGCTGCTGGCAGAGTTCCTGTCAAGGGATCAAGTCTTCCAACAGAATGGTTATGGTTT
          70                  90                 110
AACTCAGCAGAATTTGTTGAACAACTACGACATGCTGGGGATCATGGCATGGAATGCAAC
                                  M   L   G   I   M   A   W   N   A   T
         130                 150                 170
TTGCAAAAACTGGCTGGCAGCAGAGGCTGCCCTGGAAAAGTACTACCTTTCCATTTTTTA
 C   K   N   W   L   A   A   E   A   A   L   E   K   Y   Y   L   S   I   F   Y
         190                 210                 230
TGGGATTGAGTTCGTTGTGGGAGTCCTTGGAAATACCATTGTTGTTTACGGCTACATCTT
 G   I   E   F   V   V   G   V   L   G   N   T   I   V   V   Y   G   Y   I   F
         250                 270                 290
CTCTCTGAAGAACTGGAACAGCAGTAATATTTATCTCTTTAACCTCTCTGTCTCTGACTT
 S   L   K   N   W   N   S   S   N   I   Y   L   F   N   L   S   V   S   D   L
         310                 330                 350
AGCTTTTCTGTGCACCCTCCCCATGCTGATAAGGAGTTATGCCAATGGAAACTGGATATA
 A   F   L   C   T   L   P   M   L   I   R   S   Y   A   N   G   N   W   I   Y
         370                 390                 410
TGGAGACGTGCTCTGCATAAGCAACCGATATGTGCTTCATGCCAACCTCTATACCAGCAT
 G   D   V   L   C   I   S   N   R   Y   V   L   H   A   N   L   Y   T   S   I
         430                 450                 470
TCTCTTTCTCACTTTTATCAGCATAGATCGATACTTGATAATTAAGTATCCTTTCCGAGA
 L   F   L   T   F   I   S   I   D   R   Y   L   I   I   K   Y   P   F   R   E
         490                 510                 530
ACACCTTCTGCAAAAGAAAGAGTGTGCTATTTTAATCTCCTTGGCCATGTGGGTTTTAGT
 H   L   L   Q   K   K   E   C   A   I   L   I   S   L   A   M   W   V   L   V
```

MATCH WITH FIG. 1B

MATCH WITH FIG. IA

# FIG. IB

```
      550                 570                 590
AACCTTAGAGTTACTACCCATACTTCCCCTTATAAATCCTGTTATAACTGACAATGGCAC
 T  L  E  L  L  P  I  L  P  L  I  N  P  V  I  T  D  N  G  T
      610                 630                 650
CACCTGTAATGATTTTGCAAGTTCTGGAGACCCCAACTACAACCTCATTTACAGCATGTG
 T  C  N  D  F  A  S  S  G  D  P  N  Y  N  L  I  Y  S  M  C
      670                 690                 710
TCTAACACTGTTGGGGTTCCTTATTCCTCTTTTTGTGATGTGTTTCTTTTATTACAAGAT
 L  T  L  L  G  F  L  I  P  L  F  V  M  C  F  F  Y  Y  K  I
      730                 750                 770
TGCCTCCTTCCTAAAGCAGAGGAATAGGCAGGTTGCTACTGCCTCGCCCCTTGAAAAGCC
 A  L  F  L  K  Q  R  N  R  Q  V  A  T  A  L  P  L  E  K  P
      790                 810                 830
TCTCAACTTGGTCATCATGGCAGTGGTAATCTTCTCTGTGCYTTTTACACCCTATCACGT
 L  N  L  V  I  M  A  V  V  I  F  S  V  X  F  T  P  Y  H  V
      850                 870                 890
CATGCGGAATGTGAGGATCGCTTCACGCCTGGGGAGTTGGAAGCAGTATCAGTGCACTCA
 M  R  N  V  R  I  A  S  R  L  G  S  W  K  Q  Y  Q  C  T  Q
      910                 930                 950
GGTCGTCATCAACTCCTTTTACATTGTGACACGGCCTGTGGCCTTTCTGAACAGTGTCAT
 V  V  I  N  S  F  Y  I  V  T  R  P  V  A  F  L  N  S  V  I
      970                 990                 1010
CAACCCTGTCTTCTATTTTCTTGTGGGAGATCACTTCAGGGACATGCTGATGAATCAACT
 N  P  V  F  Y  F  L  V  G  D  H  F  R  D  M  L  M  N  Q  L
```

MATCH WITH FIG. IC

MATCH WITH FIG. 1B

FIG. 1C

```
      1030                  1050                  1070
GAGACACAACTTCAAATCCCTTACATCCTTTAGCAGATGGGCTCATGAACTCCTACTTTC
 R  H  N  F  K  S  L  T  S  F  S  R  W  A  H  E  L  L  L  S
      1090                  1110                  1130
ATTCAGAGAAAAGTGAGGGGCTTGTGAAACAGATTGTTCTACAGATGAATCTGTAAGCCA
 F  R  E  K  *
      1150                  1170                  1190
GTTACAGTTTGCTTTAACTCATAGACATCAATCAGAGAGTGTCACAGATTTAACCTTGAT
      1210                  1230                  1250
CTAAAGACAAGTTGTACCCAGAGTATGTGAAAAGAATGGGACGACAAGAATGTACTGGTT
      1270                  1290                  1310
TCTTCCTCTAAGAATTGAAAGGTGTTGAACTGCCTTATGTTTGGGCATGTAACTCCAAAA
      1330                  1350                  1370
TACTAGGTAGTATAAGGCTTTCTCAATCAGTGCAAAAATGGAAGATATATAAAGCAACAA
      1390                  1410
GTTGTCTGCATTTGATCACTGGTCAGATTGTAAAAAAAAAAAAAAAAA
```

FIG. 2

```
  3 GIMAWNATCKNWLAAEAALEKY.....YLSIFYGIEFVVGVLGNTI  43
    :: :|| .|... :.::.   |:          .|.: ||:  |:|:  |.:
  4 DLEPWNSTINGTWEGDELGYKCRFNEDFKYVLLPVSYGVVCVLGLCLNVV  53

 44 VVYGYIFSLKNWNSSNIYLFNLSVSDLAFLCTLPMLIRSYANGN.WIYGD  92
    .:|  ::  .||.||..|..|:|.|||   :  ..||:|:  ||.|: |  ::.
 54 ALYIFLCRLKTWNASTTYMFHLAVSDSLYAASLPLLVYYYARGDHWPFST 103

 93 VLCISNRYVLHANLYTSILFLTFISIDRYLIIKYPFREHLLQKKECAILI 142
    |||   |:::..|||.|||||| ||:.|:| :  |::.  :.:  :|  :
104 VLCKLVRFLFYTNLYCSILFLTCISVHRCLGVLRPLHSLRWGRARYARRV 153

143 SLAMWVLVTLELLPILPLINPVITDNGTTCNDFASSGDPNYNLIYSMCLT 192
    . .:|||||     |:| ::... : :. .||:| ...:  ... : || .:
154 AAVVWVLVLACQAPVLYFVTTSVRGTRITCHDTSARELFSHFVAYSSVML 203

193 LLGFLIPLFVMCFFYYKIALFLKQRNRQVATALPLEKPLNLVIMAVV..I 240
    | | :|: :: . |  :|  |  ... ....:|| .|. .: .:|:|  :
204 GLLFAVPFSVILVCYVLMARRLLKPAYGTTGGLPRAKRKSVRTIALVLAV 253

241 FSVXFTPYHVMRNVRIASRLGSWKQYQCTQVVINSFYIVTRPVAFLNSVI 290
    |.:  | |:|| |.: ... |   |:.    |   .|| |  :|||:|  ||.:
254 FALCFLPFHVTRTLYYSFR..SLDLSCHTLNAINMAYKITRPLASANSCL 301

291 NPVFYFLVGDHFRDMLMNQLRHNFKSLTSFSRWAHELLLSFRE 333
    :||:|||. :::  : .:. ... ... .. .|:  :|  . |.
302 DPVLYFLAGQRLVRFARDAKPPTEPTPSPQARRKLGLHRPNRT 344
```

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00392

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN (MEDLINE, BIOSIS, LIFESCI, EMBASE, WPIDS)
search terms: ATP, receptor?, P, extracellular

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | ABBRACCHIO et al. Purinoceptors: Are they families of P2X and P2Y purinoceptors? Pharmac. Ther. 1994, Vol. 64, pages 445-475. | 1-10, 15-16, 18-25, 27-29 |
| A | PARR et al. Cloning and expression of a human P2u nucleotide receptor, a target for cystic fibrosis pharmacology. Proc. Natl. Acad. Sci. USA. April 1994, Vol. 91, pages 3275-3279. | 1-22, 24-26 |
| X<br>---<br>A | FISCHER et al. Identificaton of potent, selective P2y-purinoceptor agonists: Structure-activity relationships for 2-thioether derivatives of adenosine 5'-triphosphate. J. Med. Chem. 1993, Vol. 36, pages 3937-3946. | 18<br>----<br>19 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be part of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>16 MAY 1996 | Date of mailing of the international search report<br>31 MAY 1996 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>CLAIRE KAUFMAN<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00392

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | US 5,385,831 A (MULVIHILL ET AL) 31 January 1995, columns 11-12. | 17 |
| A | US 5,468,613 A (ERLICH ET AL) 21 November 1995, see entire document. | 26 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/00392

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A01N 43/04; A61K 38/17, 38/19, 31/395; G01N 33/53, 33/566, 21/75; C12Q 1/68; C07H 19/16; C07K 16/28, 14/705, 2/00, 4/12, 1/00; C12N 15/63, 1/21, 5/10; C12P 21/02

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

536/23.5, 24.3, 24.33, 24.5, 27.6; 530/300, 351, 413, 388.22; 435/320.1, 69.1, 240.1, 252.3, 172.3, 7.2, 6; 436/501, 86; 514/300, 44, 2; 424/93.2

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

536/23.5, 24.3, 24.33, 24.5, 27.6; 530/300, 351, 413, 388.22; 435/320.1, 69.1, 240.1, 252.3, 172.3, 7.2, 6; 436/501, 86; 514/300, 44, 2; 424/93.2